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Formulations comprising vitamin B12, method of production and use thereof

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FORMULATIONS COMPRISING VITAMIN B12, METHOD OF PRODUCTION AND USE THEREOF

Field of the invention

The present invention relates to a particle comprising a vitamin B12-containing microbial biomass and to compositions comprising the same. The invention further relates to a method for the production of compositions comprising said particles. The invention also relates to animal feed, human food or food supplements comprising said particles.

Background of the invention

Microorganisms are known as valuable sources of a varied range of useful compounds. Several of these compounds are located either inside or are associated with the microbial cell. Generally, to recover such compounds after fermentation of the microorganisms, it is necessary to separate the compound from the microbial biomass. However, especially when said compounds are instable to isolation techniques or when the used microorganisms are microbiologically safe and food-grade, compounds are not produced in isolated form but are produced in dry formulation together with the biomass of the organism in which they are produced. Such formulations are especially suited for use as animal feed supplement. With "microbial biomass" it is here intended a microorganism-containing product resulting from fermentation, which consists of whole, preferably non-viable cells and/or cell debris.

Vitamin B12 is an important compound for humans and animals and it is an important animal feed supplement as growth enhancer. The term vitamin B12 is used to describe compounds of the cobalt corrinoid family, in particular those of the cobalamin group. In this specification the term vitamin B12 should be attributed its broad meaning so as to include all the cobalt corrinoids of the cobalamin group, which include in particular cyanocobalamin, hydroxocobalamin, methylcobalamin, 5'-adenosylcobalamin and 5'-desoxyadenosylcobalamin characterised by cyano, hydroxyl, methyl or 5'-desoxyadenosyl radical respectively. The methylcobalamin and 5'-desoxyadenosylcobalamin compounds are known to be unstable to light in isolated form and are easily transformed to

hydroxocobalamin in aqueous solution. For this reason, commercial vitamin B12 preparations consist of the stable cyanocobalamin.

Vitamin B12 is often obtained in industrial fermentation methods using microorganisms known to produce vitamin B12.

A suitable method for the production of vitamin B12 via fermentation is described in International Patent Application WO00/37699. This document describes a non-continuous fermentation method for the production of vitamin B12 wherein a strain of *Propionibacterium* is cultured in two different fermentors under anaerobic and aerobic conditions respectively in a "fill and draw" fashion. The inhibiting effect of propionic acid on growth of *Propionibacterium* in the anaerobic phase is by this method considerably reduced leading to increased biomass and increased vitamin B12 production at the end of the fermentation.

International Patent Application WO98/06868 describes a method for the preparation of compositions comprising vitamin B12 in a concentration, based on dry matter, higher than 0.1% w/w. Such compositions are produced by a method wherein microbial cells are cultured to intracellularly produce vitamin B12, after which said cells are partially lysed and/or damaged to cause release of vitamin B12 into the medium. After separation of microbial biomass from the vitamin B12-comprising liquid phase and concentration of the latter, the concentrate solution and the microbial biomass can be combined in different ratios and said mixtures spray-dried. By this method a spray-dried biomass can be obtained with a high concentration in vitamin B12.

Even though the production of biomass with a high concentration of vitamin B12 is advantageous in several ways (ease of transportation and consequent reduction of costs) a high concentration in vitamin B12 can be less desirable in some applications.

Spray-dried biomass can for example be used in animal feed. Prior to use in the production of feed a lowering of the concentration of a biomass with high concentration of vitamin B12 may be necessary. The latter is especially desirable when lower dosages of vitamin B12 in the animal diet (e.g., for poultry) are required. A possible solution to this problem could be to blend the spray-dried biomass with a solid carrier in order to reduce vitamin B12 concentration prior to mixing with other feed components. Solid carriers to be used at this purpose could be edible solid carriers based on carbohydrates, proteins, or mixtures thereof in powder form. Said blends of vitamin B12-containing biomass and solid carrier could be added to other feed components, either directly or in the form of a premix, which also contains other vitamins, minerals and/or bioactive ingredients, in order

to produce the final feed. In order to assure good distribution of vitamin B12 in the final feed compositions, especially when low dosages need to be applied, it is very important that the blends of solid carrier and vitamin B12-containing biomass are homogeneous.

Unfortunately, we have observed that blends obtained by mixing spray-dried biomass and solid carriers are inhomogeneous. Besides they may be electrostatic, dusty and not free-flowing. Some of these problems can cause inconvenience during handling of such blends at industrial scale. Free-flowing characteristics could be increased by addition of inorganic solid carriers like silica. However we have observed that the latter does not improve homogeneity of the blends. Moreover some inorganic solid carriers, like silica, may be highly dusty, hazardous especially if inhaled, and not very desirable ingredients for animal feed. Lack of homogeneity of said blends is not desirable as it can lead to inaccurate dosage of the vitamin B12 into the final premix and/or feed, with unequal distribution of the nutrient between different animals. The latter is especially disadvantageous for small animals like poultry.

Description of the invention

An object of the present invention is therefore to provide formulations of vitamin B12-containing microbial biomass and solid carrier where the above-mentioned problems are not present.

We have found that when vitamin B12-containing microbial biomass and a solid carrier are present in the same particle, blending of vitamin B12-containing biomass with solid carrier prior to mixing with the other feed components becomes superfluous and the problems caused by the above-mentioned blends are overcome.

Therefore the invention provides a particle comprising a vitamin B12-containing microbial biomass and a solid carrier.

The particles of the invention may have different morphologies. For instance, a possible morphology of the particle may be one in which the solid carrier is mainly concentrated near the centre of the particle while the vitamin B12-containing microbial biomass constitutes a sort of continuous film of coating material around it. Another possibility may be one in which distribution of respectively vitamin B12-containing microbial biomass and solid carrier is reversed, i.e. the biomass is concentrated in the centre of the particle while the solid particles are more on the outside. A third possibility

may be one in which the particle is actually constituted by a matrix of vitamin B12-containing microbial biomass in which particles of solid carrier are entrapped or vice-versa.

The size of the particles may vary, being preferably comprised between 0.2 μm and 2000 μm. The particle size is generally comprised between 10 μm and 1000 μm, preferably comprised between 20 μm and 500 μm, even more preferably comprised between 50 μm and 300 μm, most preferably comprised between 50 μm and 150 μm.

Preferably the particles of the invention have a homogeneous particle size distribution. With the wording "homogeneous particle size distribution" it is intended that the overall particle size distribution is relatively narrow, such that at least 70% w/w of the particles, preferably at least 80% w/w, more preferably 90% w/w of the particles have a particle size comprised between 20 and 500 μm, more preferably comprised between 50 μm and 300 μm.

The particles of the invention may have a vitamin B12 concentration of typically about 0.05%-5% w/w, more typically 0.1-1% w/w, usually not exceeding 10% w/w.

Typically the moisture content of the particles is comprised between 5-10% w/w.

The invention further provides a composition characterised in that it comprises particles according to the invention.

A composition essentially consisting of particles according to the invention has a homogeneous distribution of vitamin B12 on the solid carrier, is free flowing, not dusty and non-electrostatic and can be produced very economically.

With "vitamin B12-containing microbial biomass" it is intended a micro-organism-containing-product resulting from fermentation of microorganisms capable of producing vitamin B12 and cultured under conditions conducive thereof, said micro-organism-containing-product consisting of preferably non-viable, whole cells and/or cell debris comprising vitamin B12.

Vitamin B12-containing microbial biomass preferably used in the invention is obtainable from a bacterial strain of the genus *Acetobacterium, Acetobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azobacter, Bacillus, Clostridium, Corynebacterium, Escherichia, Eubacterium, Flavobacterium, Methanobacillum, Methanosarcina, Mycobacterium, Propionibacterium, Proteus, Pseudomonas, Rhizobium, Rhodopseudomonas, Salmonella, Serratia, Streptococcus, Streptomyces* and *Xanthomonas.* Preferably a bacterium is used which is safe for consumption by humans and/or animals and does not produce endo- or exotoxins. *Propionibacteria* in particular

are food-grade and satisfy these criteria. Therefore in a preferred embodiment of the invention the particle is characterised in that the vitamin B12-containing microbial biomass is from the genus *Propionibacterium*. Preferred Propionibacterium species used at this regard are *P. freundenreichii, P. theonii, P. jensenii, P. shermanii* and *P. acidipropionici*.

The solid carrier used in the particles of the invention is a particulate material or powder, preferably non-hygroscopic, which is suitable to be used in spray-drying, multi-stage and fluid bed drying techniques.

In order to be used in the above-mentioned drying techniques the solid carrier in the particles according to the invention should have low bulk density, preferably a density comprised between 400 and 1200 kg/m^3, preferably between 400 and 1000 kg/m^3, more preferably about 500 kg/m^3, and preferably a particle size equal or lower than 500 μm, preferably equal or lower than 300 μm, generally comprised between 10-300 μm, more preferably comprised between 10-200 μm, most preferably comprised between 30-150 μm.

Generally the solid carrier has a moisture content of 2-15% w/w as a powder.

Preferably the solid carrier is a carbohydrate, a protein, or a mixture thereof. Suitable solid carriers are powders of casein, whey, milk, maltodextrin, corn steep solids, starch, edible flour, or mixtures thereof. Most preferably the solid carrier is edible flour. With "edible flour" it is intended a finely ground meal (essentially consisting of starch and protein) obtainable from edible cereal grains or seeds (e.g. wheat, rice, maize, barley, oat, rye, etcetera), from legumes (e.g. beans, peas, etcetera) or from edible tubers or fruits like potato's, banana's, etcetera, or a mixture thereof. Edible flour has the advantage of being cheap, light and of being a desirable component in animal feed.

In a preferred embodiment, the particle of the invention is characterised in that the weight ratio between the vitamin B12-containing microbial biomass and the solid carrier is between 0.2-5, preferably between 0.25-4, more preferably between 0.5-2.

The particles according to the invention can be produced according to any method suitable to the formation of composite particles, like spray-drying, fluid bed drying, multi-stage drying. Spray-drying, fluid bed drying and multi-stage drying techniques are known to those skilled in the art.

Preferably the particles of the invention are produced by spray-drying or multi-stage drying techniques wherein a liquid suspension of vitamin B12-containing microbial biomass is spray-dried in the presence of a solid carrier in powder form. When referred to the method of the invention, the terms spray-drying or spray-dryer are used in a broad

sense, to cover both pure spray-drying or spray dryer and multi-stage drying or multi-stage dryer.

Therefore the invention provides a method for the production of particles comprising vitamin B12-containing microbial biomass and a solid carrier, wherein a liquid suspension of vitamin B12-containing microbial biomass and a solid carrier in powder form are conveyed into the drying chamber of a spray-dryer through separate streams, wherein said liquid suspension and said solid carrier come into contact inside the spray-dryer chamber.

The vitamin B12-containing microbial biomass used in the method of the invention is preferably obtainable in industrial fermentation processes using microorganisms known to produce vitamin B12. These include bacteria belonging to the bacterial strains mentioned above. Several fermentation methods suitable to the microbial production of vitamin B12 are known to those skilled in the art. Preferably, the vitamin B12-containing microbial biomass is obtainable from a bacterial strain of the genus *Propionibacterium*. Several methods are known in the art for the fermentation of *Propionibacterium* strains under conditions conducive to the production of vitamin B12. An example is described in International Patent Application WO00/37699.

Generally the microbial cells containing vitamin B12 are concentrated and optionally purified at the end of the fermentation by one or more methods suitable to this purpose (e.g. evaporation, ultrafiltration, diafiltration, etc.).

Preferably a concentration (based on dry matter) of microbial biomass in the liquid suspension is obtained which allows economical spray-drying operation. In a preferred embodiment the liquid suspension of vitamin B12-containing microbial biomass used in the method according to the invention has a concentration of 50-300 g/l, preferably 100-300 g/l, more preferably 200-300 g/l based on dry mass per litre of concentrate.

Optionally it is possible to use a vitamin B12-containing microbial biomass with a relatively low concentration, for example of about 120-150 g/l, and further concentrate the liquid suspension, for example up to about 220-300 g/l, just prior to spray-drying. A concentrator/evaporator positioned upstream to the spray-dryer can be used for this purpose.

It is known that carboxylic acids with a low molecular weight like acetic and propionic acid are produced during fermentation of *Propionibacterium* strains. The latter represents a problem as the presence of acids during spray-drying of the corresponding microbial biomass can cause difficulties in the drying process and stickiness of the end

product. Therefore the liquid suspension of *Propionibacterium* microbial biomass is generally treated prior to spray-drying or multi stage drying, for example by applying a diafiltration step, to reduce the acid concentration to a desirable value.

Optionally the vitamin B12-containing microbial biomass is pasteurised prior to spray-drying.

In the method of the invention a solid carrier can be used as already described.

The method for the production of the particles according to the invention can be performed on a conventional spray-dryer or multi stage dryer. This type of equipment is generally used in many applications, e.g. in the dairy industry.

Generally a spray-dryer or multi-stage dryer comprises at least a drying chamber, with a distribution element for atomising liquid to be spray-dried, means for supplying drying gas and means for discharging the spray-dried product from the device. Besides the above-mentioned elements, a multi stage-dryer further comprises one or more fluidised beds. A spray-dryer or multi-stage dryer suitable to be used in the method according to the invention should further be furnished with means suitable to supply solid carrier into the dying-chamber.

Therefore a spray-dryer or multi-stage dryer suitable for being used in the method of the invention comprises at least two product-inlet ports, generally positioned on the upper part of the spray-dryer chamber. Through one inlet port the liquid suspension of microbial biomass is atomised and conveyed into the spray-dryer chamber. Said inlet port is furnished with means suitable to atomise the liquid suspension (e.g. nozzle, rotating disk atomisers etc.). The second product-inlet port is used to convey the solid carrier, generally in powder form into the drying chamber; however a slurry might also be applicable. Optionally the spray-dryer may comprise means for the recovery of fine particles. Said particles can be reintroduced into the drying chamber by means of a third product-inlet port or into the pipeline conveying either the microbial biomass or the solid carrier into the system. Optionally the spray-dryer is part of a multi-stage dryer comprising one or more fluidised beds.

Typically an inlet temperature of the air in the drying chamber of the spray-dryer is used which is between 120-250°C, preferably between 160-220°C. The outlet temperature of the air is generally comprised between 60-90°C.

Both streams of solid carrier and atomised liquid suspension of microbial biomass are conveyed into the drying chamber. The stream of droplets produced by atomisation of the liquid suspension of microbial biomass comes into contact with the solid carrier in

powder form into the drying chamber so as to produce particles comprising vitamin B12-containing microbial biomass and the solid carrier.

The method of the invention advantageously allows adjustment of the amount of vitamin B12-containing microbial biomass on the solid carrier in order to assure an optimal distribution of vitamin B12 containing biomass on the particle. Advantageously the invention also allows adjustment of the amount of vitamin B12 on the solid carrier, depending both on the content of vitamin B12 in the microbial biomass and on the final application of the resulting particles.

Preferably the weight ratio between the vitamin B12-containing microbial biomass and the solid carrier used in a method of the invention should be between 0.2-5, preferably between 0.25-4, more preferably between 0.5-2.

With a method of the invention particles can be produced as already described. An advantage related to the method of the invention is that particles with a homogeneous mean particle size distribution are obtained. Another advantage in the method according to the invention is that the spray-drying step allows production of "pasteurised" compositions comprising the particles according to the invention. This is especially advantageous when certain types of solid carrier, which are not always free of microorganisms and yeast (e.g. edible flour) are used.

The invention provides particles comprising a vitamin B12-containing microbial biomass and a solid carrier obtainable by a method of the invention. Said particles have the desirable characteristics already described above.

The particles of the invention can be used as or in the production of animal feed. To this end, the particles containing vitamin B12 are added to other feed components, either directly or in the form of a premix, which may also contain other vitamins, enzymes, minerals and/or bioactive ingredients.

Therefore the invention provides an animal feed comprising particles according to the invention.

Feeding an animal a diet comprising a feed according to the invention promotes its growth.

Thus the invention also provides the use of an animal feed according to the invention to promote the growth of an animal.

The particles of the invention can also be used in the production of a human food or food supplement. Therefore the invention provides a human food or food supplement comprising particles according to the invention.

The invention will now be illustrated by way of examples which however are not intended as to be limiting.

Examples

General methods

Fermentation broth from *Propionibacterium freudenreichii* CBS 929.97 was obtained as described in International Patent Application WO00/37699.

The fermentation broth was concentrated by means of ultrafiltration (on polysulfon MW cut off 5-10 kD, Koch HFK 151 VSV) or microfiltration (on Membralox ceramic 0.1μm) up to a biomass concentration of 100-150 g/l.

After ultrafiltration or microfiltration, the propionic acid in the biomass had a concentration of about 25-30 g/l. To reduce the concentration of propionic and acetic acid in the biomass, the biomass concentrate was diafiltered with water. This diafiltration was performed by an in-line addition of water to the concentrate at the same rate as the permeate flow. The diafiltration was stopped at a propionic acid concentration lower than 5 g/l. At this purpose a ratio (v/v) water : concentrate of 3-4 : 1 was applied.

After diafiltration the concentrated biomass was pasteurised during 1 minute at a temperature of 90-94°C (either by direct steam injection or heating by a plate heat exchanger).

The pasteurized biomass was further concentrated by a multistage (vacuum) falling film evaporator with vapor recompression. This type of evaporator is known to those skilled in the art.

The following conditions were applied.

Biomass feed rate	2000-3000 l/h (corresponding to 300 kg dry matter/h)
Pre-heater temperature	92°C
1st stage temperature	65-70°C
5th stage temperature	50-55°C
Temperature of concentrate	45-50°C
Biomass concentration	22-26% (1250 kg/h)

The biomass concentrate was spray-dried on a Multi Stage Dryer (NIRO AS, Denmark).

The following set up was used in all the experiments.

The vitamin B12-containing biomass was fed into the drying chamber by a nozzle with a biomass feed rate of 1250 kg dry matter/h).

Nozzle pressure	190-195 bar
Air inlet temperature (co current)	200-220°C
Air outlet temperature	75-92°C
Air Internal fluid bed temperature	55-60°C
Air 1st external fluid bed temperature	30-35°C
Air 2nd external fluid bed temperature	15-20°C
Powder temperature	< 30°C

Fines were returned via a cyclone to the nozzle area.

Example 1

In this example vitamin B12-containing biomass was spray-dried in absence of solid carrier applying the above-mentioned spray-drying conditions.

Example 2

In this example 600 Kg of vitamin B12-containing spray-dried biomass obtained in Example 1 was mixed in an external powder mixer (batch) with 300 kg of wheat flour and 1 kg of silica (Aerosil 200®).

Example 3

In this example 120 g $MgSO_4.7H_2O$ per kg of concentrate was added to the diafiltered biomass concentrate (120 g/l biomass concentration) before evaporation. The mixture was evaporated to a dry matter content of 32% and spray dried as described above.

Example 4

In this example vitamin B12-containing biomass was spray-dried in presence of wheat flour as a solid carrier applying the above-mentioned spray-drying conditions. The wheat flour was dosed as a powder at a rate of 180-220 kg/h. Both streams of solid

carrier and atomised liquid suspension of microbial biomass were separately conveyed into the spray dryer chamber. The powder was dosed into the spray dryer chamber close to the area of the nozzle feed stream.

Example 5

The characteristics of the compositions comprising vitamin B12-containing spray-dried biomass obtained in examples 1 to 4 were analysed and are reported in the following table.

Example	1	2	3	4
Vitamin B12 content (mg/kg)	1600	1080	985	1110
Dry matter (% w/w)	94	94	86	94
Presence of lumps	no	no	yes	no
Dust (mg/kg)	50	10500	410	320
Flowability	ok	ok	ok	ok
Particles (% w/w) with particle size lower than 300 μm)	n.r.	91	99	82
Total plate count per g	20	14000	20	500
Moulds per gram	100	500	10	20
Visual homogeneity	yes	no	no	yes

Visual homogeneity in the context of the present table means that the distribution of microbial biomass on the solid carrier is visually homogeneous, i.e. no separation is observed between spray-dried microbial biomass and solid carrier.

By comparing the results obtained in example 4 with those obtained in the other examples it is clear that compositions essentially consisting of the particles of the invention are homogeneous, not hygroscopic, free-flowing, not dusty and almost free of moulds and bacteria.

CLAIMS

1. Particle comprising a vitamin B12-containing microbial biomass and a solid carrier.
2. The particle of claim 1 wherein the weight ratio between the vitamin B12-containing microbial biomass and the solid carrier is between 0.2-5, preferably between 0.25-4, more preferably between 0.5-2.
3. The particle of claim 1 or 2 wherein its particle size is comprised between 0.2 μm and 2000 μm, preferably comprised between 10 μm and 1000 μm, more preferably comprised between 20 μm and 500 μm, even more preferably comprised between 50 μm and 300 μm, most preferably comprised between 50 μm and 150 μm.
4. The particle of any one of claims 1 to 3 wherein the vitamin B12-containing microbial biomass is from a bacterial strain of the genus selected from the group of *Acetobacterium, Acetobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azobacter, Bacillus, Clostridium, Corynebacterium, Escherichia, Eubacterium, Flavobacterium, Methanobacillum, Methanosarcina, Mycobacterium, Propionibacterium, Proteus, Pseudomonas, Rhizobium, Rhodopseudomonas, Salmonella, Serratia, Streptococcus, Streptomyces* and *Xanthomonas.*
5. The particle of any one of claims 1 to 4 wherein the solid carrier is a carbohydrate, a protein, or a mixture thereof.
6. The particle of claim 5 wherein the solid carrier is selected from a powder of casein, whey, milk, maltodextrin, corn steep solids, starch, edible flour, or a mixture thereof.
7. The particle of claim 6 wherein the edible flour is obtainable from wheat, rice, barley, maize, oat, rye, potato, banana, or a mixture thereof.
8. Method for the production of particles comprising vitamin B12-containing microbial biomass and a solid carrier, wherein a liquid suspension of vitamin B12-containing microbial biomass and a solid carrier in powder form are conveyed into the drying chamber of a spray-dryer through separate streams, and said liquid suspension and said solid carrier come into contact inside the spray-dryer chamber.
9. The method of claim 8 wherein the weight ratio between the vitamin B12-containing microbial biomass and the solid carrier is between 0.2-5, preferably between 0.25-4, more preferably between 0.5-2.
10. The method of claim 8 or 9, wherein the solid carrier has a particle size equal or lower than 500 μm, preferably equal or lower than 300 μm, more preferably comprised

between 10-300 µm, even more preferably comprised between 10-200 µm, most preferably comprised between 30-150 µm.

11. The method of any one of claims 8 to 10 wherein the vitamin B12-containing microbial biomass is obtainable from a bacterial strain of the genus *Propionibacterium*.
12. The method of any one of claims 8 to 11 wherein the solid carrier is a carbohydrate, a protein, or a mixture thereof.
13. The method of any one of claims 8 to 12 wherein the solid carrier is edible flour.
14. The method of any one of claims 8 to 13 wherein the concentration of vitamin B12-containing microbial biomass in the liquid suspension is between 50-300 g/l, preferably 100-300 g/l, more preferably 200-300 g/l based on dry matter.
15. Particle comprising vitamin B12-containing microbial biomass and a solid carrier obtainable by the method according to any one of claims 8 to 14.
16. Composition comprising particles according to any one of claims 1 to 7 or according to claim 15.
17. An animal feed comprising particles according to claim 1 to 7 or according to claim 15.
18. Human food or food supplement comprising particles according to claim 1 to 7 or according to claim 15.

FORMULATIONS COMPRISING VITAMIN B12, METHOD OF PRODUCTION AND USE THEREOF

ABSTRACT

The present invention describes particles comprising a vitamin B12-containing microbial biomass and a solid carrier and compositions comprising said particles. Compositions essentially consisting of particles according to the invention are characterised by a homogeneous distribution of vitamin B12 on the solid carrier, are free flowing, not dusty and non-electrostatic and can be produced very economically. The invention further describes a method for the production of compositions comprising said particles. According to this method a liquid suspension of vitamin B12-containing microbial biomass and a solid carrier in powder form are conveyed into a drying chamber of a spray-dryer through separate streams, wherein said liquid suspension and said solid carrier come into contact inside the spray-dryer chamber. The invention also discloses the use of said particles for the production of animal feed and human food.